

Assessment of Testicular Toxicity in Laboratory Animals

by Ralph Heywood* and Ronald W. James*

The currently available methods of assessing testicular toxicity and fertility are discussed. Compounds inducing testicular atrophy mainly affect the seminiferous epithelium, with progressive loss of spermatozoa, spermatids, spermatocytes, and spermatogonia. Quantitative histometric analysis of spermatogenesis, together with hormone assays, could be used to advantage in defining the underlying mechanisms of toxicity.

Introduction

The conventional chronic or long-term animal toxicity study is a key feature in the assessment of the safety of chemicals. One may distinguish between its use for the predictive evaluation of new compounds and its incorporation in a scheme designed to help lessen or clarify a recognized hazard. It is required that these studies be carried out in two species, a rodent and a nonrodent. Animal studies are essentially comparative, in that the spontaneous and induced pathological manifestations are compared in control and test animals, respectively. It is essential to determine whether the lesion or finding is attributable to the test compound and if so, whether its incidence is dose-related. The most important interpretative factors are careful observation of the living animal, assessed by veterinary clinical techniques, adequate instrumentation, and detailed pathological investigations.

The techniques which are available for the investigation of testicular function apply mainly to the dog. With the dog, several investigations are possible. The length and breadth of the testes can be measured through the scrotal sac using calipers; the formula $\pi lb/4$, where l is length and b breadth, is used to calculate the cross-sectional area of the sagittal plane. This simple technique gives a good indication of the rate of development of the testes. Semen samples can be obtained from the dog with comparative ease by using an artificial vagina (*1*). The semen collected can be measured and investi-

gated by standard methods. The mean volume of the ejaculate of the dog is 3.1 ml. The three fractions of ejaculate cannot be differentiated, particularly with respect to the first and second fractions. The volume of the first fraction is small and flows slowly over the artificial vagina, so that the first two fractions are invariably mixed. The concentration of the sperm is not a particularly useful measurement, because the variation in volume of the third fraction can cause marked dilution of the spermatozoa. The total sperm count per ejaculate is a more meaningful measurement; it shows considerable monthly variation and is usually between 89.4×10^6 and 261.8×10^6 .

With the rat, one is limited to inspection and palpation of the gonads. If counts of epididymal spermatozoa are required, the rat must be killed. The ductus deferens is then flushed with 1 ml of 0.9% sodium chloride, and the caput, corpus, and cauda of the epididymis are macerated with 2.0, 1.0, and 2.0 ml of 0.9% sodium chloride (NaCl) respectively. The motility of the sperm can be assessed and counts made by using the hemocytometer counting chamber.

The subhuman primates used in toxicological studies are usually young adolescent and sexually immature animals, which makes investigation of testicular function a major problem.

The biochemical investigations available are inappropriate to the measurement of testicular function. Investigation of seminal fructose levels could, however, be of interest. It is possible to measure circulating plasma hormones, notably FSH, LH, prolactin, and testosterone, but the value of these investigations in toxicological studies depends on

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the establishment of baseline data. In the beagle dog, plasma testosterone levels are about 600 ng/ml. In the monkey, plasma testosterone levels should be critically reviewed, as considerable seasonal variations have been demonstrated in this species (2).

Macroscopic postmortem examination rarely reveals any significant pathological changes. Organ weight analysis is an extremely useful parameter. In the rat, all organs grow more slowly than the body, except for the testes and prostate. The testes grow at the same rate as the body, while the prostate develops faster. With increasing age, there is a decrease in the gonad weights. In the dog, a linear positive correlation is found for all organs except the thymus (3). With the exception of the testes and heart, the organs show negative allometric growth.

However, positive allometric growth is observed for the testes within the age range 7–180 days. The testes of the monkey show a marked increase in weight, beginning at about three years of age, after the adolescent growth spurt, and continuing until five years of age.

Histopathological examination of tissues is of crucial importance. Zbinden (4) includes testes and epididymis in his list of first priority tissues and organs to be examined. The interpretation of histopathological findings must be made with reference to the recognized common pathology for the species employed, the changes predictable by virtue of the pharmacological action of the compound, and manifestation of novel and unpredicted response.

In the rat, age change can be detected in the testes of rats living to two years of age; about 20%

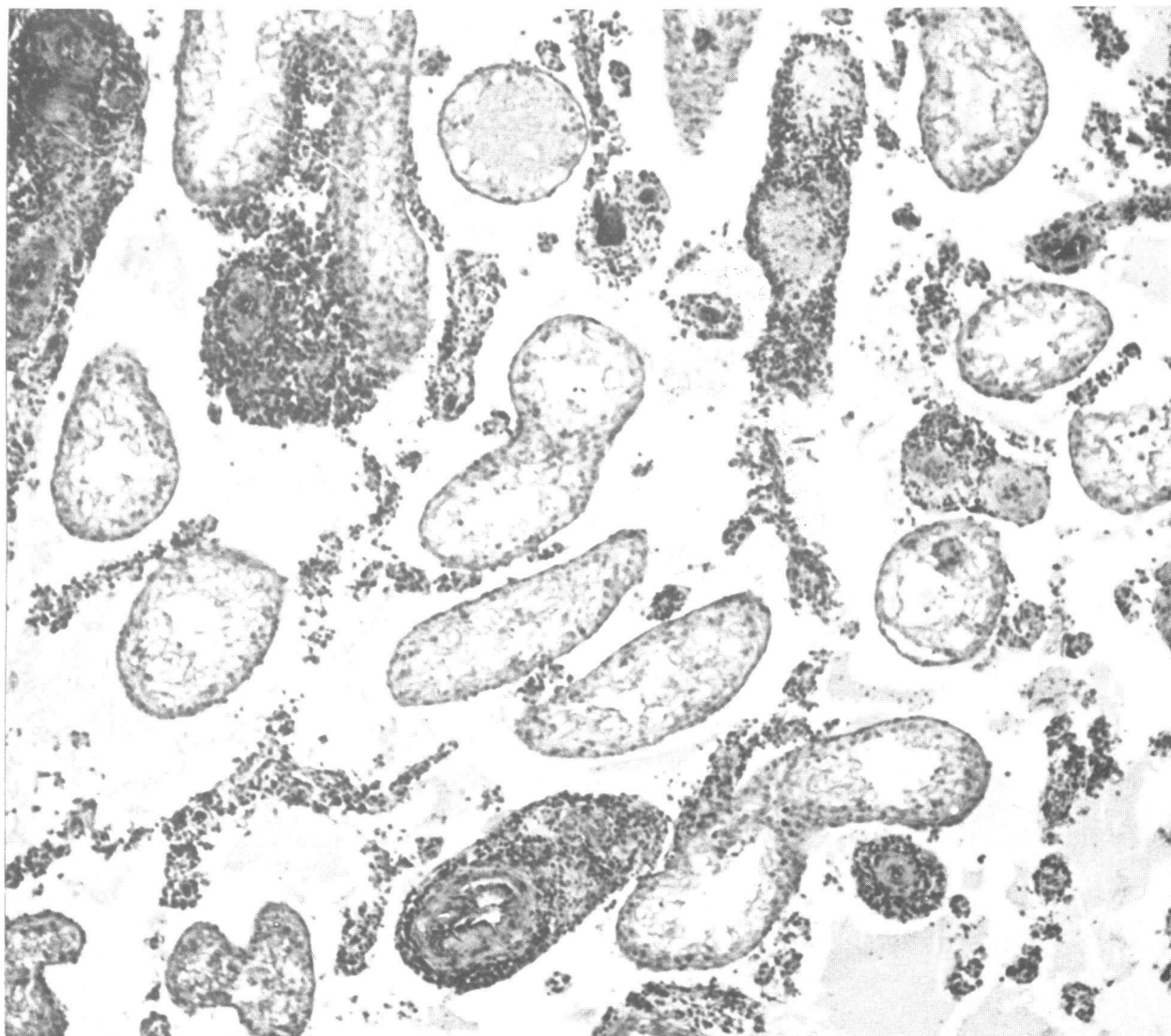


FIGURE 1. Testicular atrophy of age, rat. $\times 62.5$.

of such animals show some degree of unilateral or bilateral atrophy; 8% show atrophy with periarteritic change in the spermatic artery; and in approximately 2% of animals one can expect to find interstitial cell hyperplasia (Fig. 1). Testicular toxicity in the rat usually shows as an arrest or impairment of spermatogenesis, usually accompanied by some atrophic change in the prostate or seminal vesicles. Usually, neither the Sertoli nor the Leydig cells are involved. Chemically induced testicular atrophy is illustrated in Figure 2. The findings are consistent

with those reported by Ribelin (5). It is frequently suggested that reduction in food intake, together with suppression of body weight gain is of importance in testicular atrophy. However, in my experience, these factors are of minimal importance in the aetiology of testicular atrophy in the rat: body-weight gain can be suppressed by up to 45% for 78 weeks without significantly affecting the incidence of testicular atrophy. Rather unusual manifestations of testicular toxicity in the rat are the spermatic granulomata, which appear as soft yellow bilateral

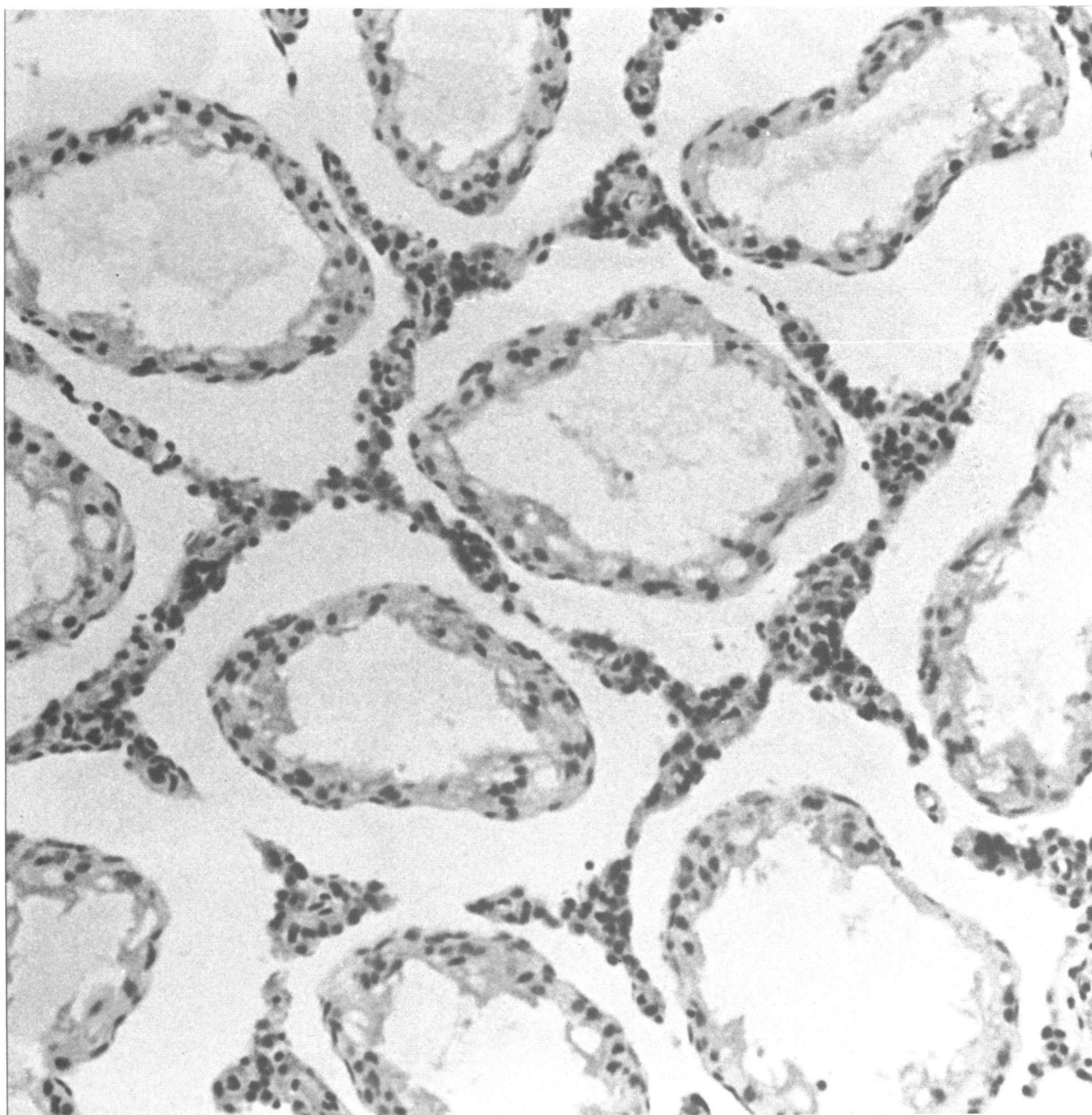


FIGURE 2. Testicular atrophy, drug-induced, rat. $\times 125$.

bulges within the tunica of the cauda epididymis (Fig. 3). The presence of myeloid bodies in the epididymal tissues is another unusual manifestation of toxicity in the rat (Fig. 4). These bodies are formed from autophagic vacuoles and contain lyso-

somes in which the membranous elements are inadequately digested. Drug-induced inclusion bodies in the epididymis could be detrimental to proper sperm maturation and storage.

Toxicological studies in the dog usually last for up

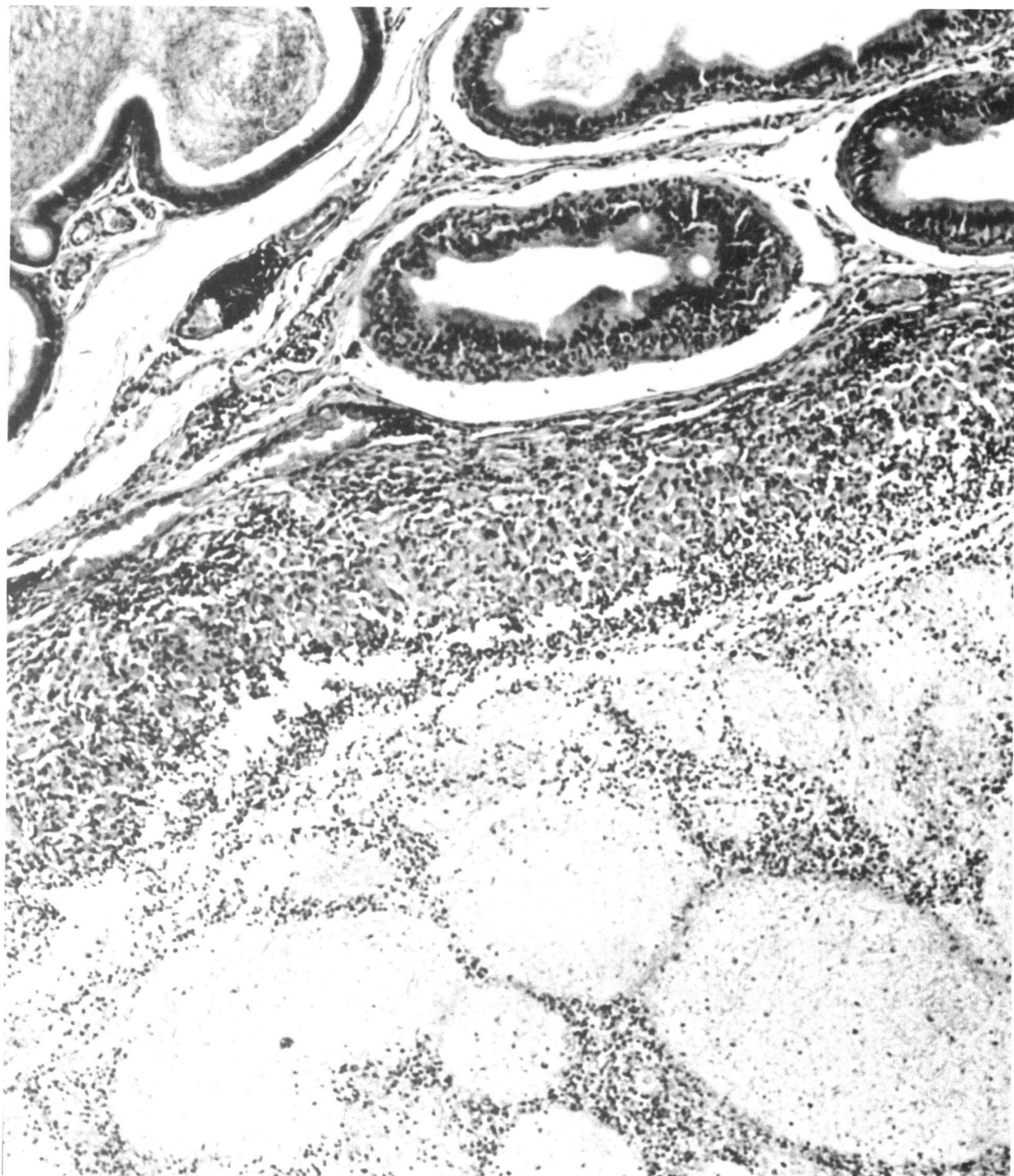


FIGURE 3. Spermatocytic granuloma, rat. $\times 62.5$.

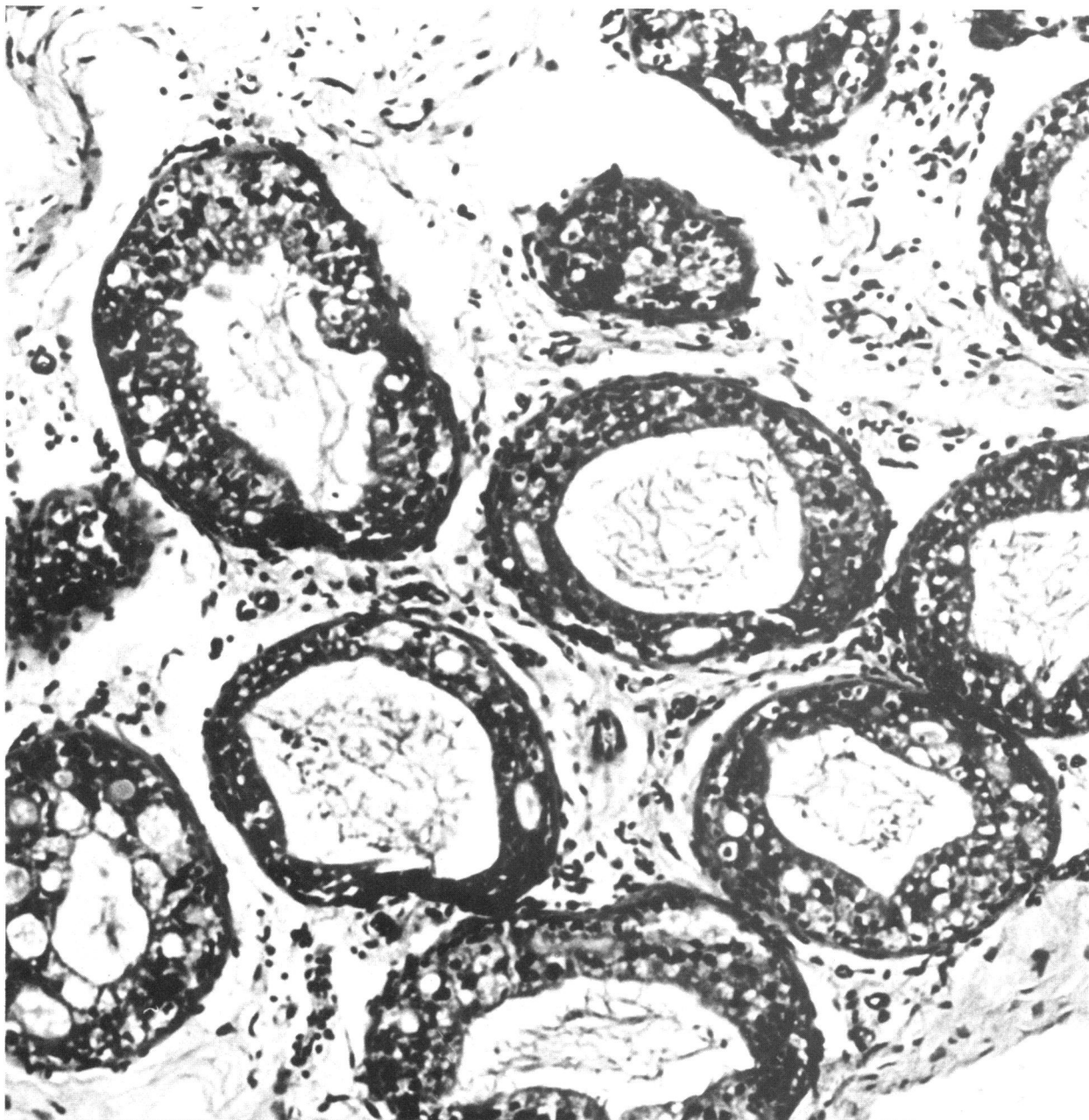


FIGURE 4. Epididymal vacuoles, rat. $\times 160$.

to two years, at which time the testicles do not show any age-induced changes. In dogs reaching eight years or more, 11% of animals are found to have tumor cells, interstitial cell adenomas, or Sertoli cell tumors in the testes. A further 9% of animals show varying degrees of hyperplasia of the interstitial cells. The histopathological picture of toxic change is one of arrest at varying stages of spermatogenesis, accompanied by some changes in the prostate.

With subhuman primates, we have seen only one

compound which affected the testes, and this induced a degree of testicular atrophy.

It is desirable that an attempt should be made to quantitate testicular changes in toxicological studies. This could be achieved by the classification of the tubules according to the stages of spermatogenesis, with enumeration of the germ cells present. The diameter of the tubules could be measured. The ratio of germ cells to Sertoli cells might prove to be a useful index of testicular toxicity.

Compounds Inducing Testicular Toxicity

The testes are not common target organs for toxicity. Gomes (6) has reviewed chemical agents affecting male fertility. Compounds affecting the testes may have direct toxicity, by acting on a particular stage of spermatogenesis or on the accessory sex glands, or an indirect effect, by acting on the interstitial cells or on the adenohypophysis. It is possible to classify compounds by their mode of action on the testes.

Steroid Hormones

Inhibition of spermatogenesis by steroid compounds is probably caused by direct action on spermatogenesis and also by action on those cells in the endocrine glands which secrete the hormones essential to normal development. This group of compounds includes estrogens and progestagens, compounds used for regulating fertility such as methallibure and clomiphene, and testosterone. High dosage levels of methyl testosterone (10 mg/kg-day and above) suppress spermatogenesis in the rat, but methyl testosterone has no effect on spermatogenesis in the dog when administered at levels up to 6 mg/kg-day (7).

Neuroleptics

Neuroleptics produce a variety of endocrine and metabolic effects. Reserpine affects testicular activity in the rat. Interstitial tissue atrophy and also atrophy of the prostate and seminal vesicles occur. The phenothiazines and the butyrophenones induce endocrine effects in the female rat, but do not affect the testes in the male (8). In the dog, phenothiazines cause low testicle weights, particularly at high dosage levels, but no morphological change is induced. The benzamide derivatives tend to induce changes in male and female dogs and rodents. The endocrine effects of these neuroleptics are probably attributable to effects on the hypothalamic nuclei.

Alkylating Agents

Alkylating agents act on dividing or developing cells by affecting DNA synthesis. The aziridines, procarbazine and busulphan, are examples of such compounds, most of which exert their effect on the spermatogonia.

Metals

Cadmium is a metal known to affect spermatogenesis in several species; it is suggested that the action is on DNA synthesis.

Compounds Lowering Blood Pressure

Adrenergic neurone-blocking agents, particularly the guanethidine derivatives (9) and other compounds that reduce hypertension or cause vasodilation can induce spermatocele granuloma in rodents. The granulomata are due to leakage of sperm into the interstitial tissue, following rupture of the vas deferens at the origin of its straight portion. Rupture is attributable to interference with seminal emission from the cauda epididymis.

Compounds Inducing Phospholipidosis

Anorectics, hypocholesterolemic agents, and antihistamines can induce cytoplasmic vacuoles filled with multicentric myeloid bodies in the epididymal epithelium.

Cyclohexylamine

Cyclohexylamine is a metabolite of the cyclamate sweeteners and has been shown by Gaunt et al. (10) to induce suppression of spermatogenesis in the rat.

Other Compounds

A variety of compounds are cited by Newburgh (11) as affecting spermatogenesis; these include phenacetin in rats, hexachlorophene in rats, fluoroacetamide in rats, Dieldrin in mice, and DDT in rats. To this list could be added analgesics, antitussives, chlorhydrins, vinca alkaloids, and many others.

Fertility Studies

If the results of a toxicological study indicate a possible effect on male fertility, the reproductive function can be studied by a variety of techniques.

The sensitivity of germinal cells to the harmful agents increases as differentiation proceeds to the spermatid stage. Spermatids are sensitive to mutagenic effects, spermatozoa are somewhat less sensitive, and spermatogonia are the least sensitive. Some compounds, particularly the alkylating agents, produce their effect in the divisional stages, and the spermatogonia are more sensitive than the spermatocytes.

The effects of agents affecting spermatogenesis

Table 1. Cell types affected at time of mating.

Week	Cell type	Division state
1	Epididymal spermatozoa	Development and maturation
2		
3		
4		
5	Testicular sperm and spermatids	2nd Meiotic division
6		
7		
8		
9	Spermatocyte	1st Meiotic division
10		
11		
	Spermatogonia	Mitosis

are apparent after varying periods, depending on the type of cells affected (Table 1). If epididymal sperm are damaged, the semen is affected soon after the administration of the toxic compound, but if the effect is on the spermatogonia, the damaged cells will not be apparent for several weeks after the damage has been done.

Antifertility compounds are tested mainly by mating untreated female animals with treated males. The type of damage produced is related to the change in sperm quality. Death of cells causes a decrease in the number of sperm. Abnormal spermatogenesis may lead to morphological defects, and damage to the genetic apparatus, which could result in embryonic death or teratogenicity.

The most useful study is a fertility study in the rat. The compound under investigation is administered by the route most applicable to human exposure at three dosage levels. The male rats only are treated for 63 days prior to mating and during the 20-day mating period. The high dosage level should be selected as the one which just causes, or fails to cause, the minimal adverse reaction in the parent animals. The low dosage should show some relationship to the likely human exposure, whilst the intermediate dosage should be logarithmically between the high and low dosage levels. Twelve treated males and 24 untreated females are required for each group. If the compound is given by intragastric intubation, a standard dosage volume of 1 ml/100 g body weight is preferred. The control animals are dosed with the vehicle. Treatment of the males commences when the animals are 42 days of age and is continued through the 63 days prior to mating and during the 20-day mating period. During this predosing period, clinical signs are noted, and the body weight is recorded. At mating, one male is caged with two untreated females. Vaginal smears are taken daily, and the day when sperm are present is considered to be day 0 of gestation. On day 20 of gestation, ten females per group are sacrificed for

examination of the ovaries, uterus and contained fetuses. The number of viable young, early and late resorptions, implantations and corpora lutea are counted for each female; pre- and post-implantation losses are subsequently calculated. Each litter is weighed and the mean pup weight determined. All fetuses are examined for various external abnormalities. Although not routinely processed further, one half of each litter is preserved in Bouin's solution and the other half preserved in alcohol, against the contingency of serial visceral sectioning (Wilson technique) or clearing and staining of the skeleton being indicated. The remaining 14 females are allowed to give birth and rear young for 21 days. The length of gestation is recorded, effects on parturition and subsequent lactation become apparent, the number of dead and live young, percentage litter loss, abnormal young, litter and mean pup weights are determined for each litter at birth, and subsequently on days 4, 12 and 21 post-partum. Both dam and litter are sacrificed on day 21 post-partum and subjected to gross autopsy. The results of these studies are statistically analyzed on a litter basis. The type of study obviously can be modified by altering the dosing period or by investigating reversibility of drug effect. The period of administration of the compound to the male may give rise to considerable practical problems, such as those involved in continual long-term intravenous injection. Also of importance is the fact that a rat can develop detoxification mechanisms with remarkable rapidity by enzyme induction and male rats dosed for this nine-week period might be so affected.

If a mutagenic effect is suspected, a dominant lethal assay can be made. The dominant lethal assay should be complemented by teratogenicity studies and multigeneration or other mutagenic assays. The usual protocol is to use groups of 20 males, treated with three dosage levels of the compound, compared with a control group. The compound is usually administered on five consecutive days by the intended human route. Two days after the fifth dose, the test males are paired for one week on a one-to-one basis with mature untreated females. Subsequently, new batches of untreated females are caged with the test males during the second, third and fourth weeks. Further batches of females can be mated if premeiotic stages of development are to be covered. Fourteen days after sperm have been discovered in the vagina, the females are killed and examined for corpora lutea, implantation sites, viable embryos and early and late embryonic deaths. The pre- and post-implantation losses are calculated and analyzed.

A variety of other tests have been devised, such as tests to study the fertility and general reproduc-

tive performance, but in these studies the males are given a pretreatment period of 63 days, while females are treated for 14 days. The multigeneration studies are carried out in males and females, each given the compound under test for 60 days prior to mating, the treatment being continued throughout the study for all generations. These studies are extremely useful in the assessment of male fertility, but make the separation of male and female effects very difficult.

Conclusion

The testis is an organ which has received scant attention from the toxicologist. Techniques are available to investigate pathological, endocrinological, and biochemical changes, and testicular function can be investigated by a variety of mating studies. These studies would not only define morphological and biochemical changes, but also the mechanisms of their induction. Research into the basic problems of testicular toxicity would be rapidly rewarded.

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